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Cloning of the *Vibrio harveyi* luciferase genes: Use of a synthetic oligonucleotide probe

(bacterial luciferase/mixed-sequence oligonucleotide)

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ABSTRACT **A mixed-sequence synthetic oligonucleotide probe was used to isolate a clone containing the gene encoding the α subunit of bacterial luciferase from *Vibrio harveyi* and part of the gene coding for the β subunit. DNA sequence analysis has allowed us to determine that the genes are closely linked on the bacterial chromosome and transcribed in the same direction. Comparison of the sequences in the regions preceding the two structural genes has revealed considerable homology and has identified sites that may be involved in the expression of the genes. Identification of a clone from a clone bank of total genomic DNA from this organism shows that mixed probes can be successfully used to isolate a gene of interest from any bacterium provided some protein sequence for the gene product is available.**

The light-emitting reaction of the marine bioluminescent bacterium *Vibrio harveyi* is catalyzed by bacterial luciferase, an α–β dimer that has subunit molecular weights of 42,000 and 37,000, respectively (1). Synthesis of luciferase and concomitant bioluminescence is inducible, resulting in an up to 10,000-fold increase in light emission per cell (2). Biochemical and physiological data indicate that control of bioluminescence in this bacterium is complex and suggest that at least some of the regulation occurs at the transcriptional level (3, 4). The amino-terminal sequences of the two subunits show considerable homology, and it has been suggested that they may have arisen by gene duplication (5). We were interested in cloning the luciferase genes to study their structure and organization and to generate specific hybridization probes with which to measure gene expression.

Recently, synthetic mixed-sequence oligonucleotides have been used to identify DNA fragments carrying genes of interest from cDNA clone banks (6) and to characterize particular eukaryotic mRNAs (7). Partial protein sequence data for luciferase were used in the design of a mixed-sequence oligonucleotide probe complementary to all possible mRNAs encoding the corresponding portion of the α subunit. This probe was used to identify a λ Charon 13 derivative carrying the entire α-subunit gene and the 5′ end of the β-subunit gene in a clone bank of *V. harveyi* DNA.

MATERIALS AND METHODS

Bacterial Strains, Cloning Vehicles, and Media. *Escherichia coli* strain ED 8654 (*sup*E *sup*F *met hsd*R$^-$ *hsd*M$^+$) (8) was used for propagation of bacteriophage λ and recombinant plasmids. The M13 cloning vehicles mp7 and mp8 were grown on *E. coli* strain JM 103 (*lac pro sup*E *thi str*A *end*A *sbc*B15 *hsd*R4 F *tra*D36 *pro*AB *lac*I^{q}ZM13) (9). DNA for cloning was isolated from *Vibrio harveyi* strain B392. Charon 13 was constructed by Blattner *et al.* (10). Plasmid pACYC184 was constructed by Chang and Cohen (11). *E. coli* was grown in either L broth or NZ broth (10). *V. harveyi* was grown in L broth supplemented with NaCl to a final concentration of 2% (LM broth).

Oligonucleotide Synthesis. A family of eight oligonucleotides was simultaneously synthesized by the solid-phase phosphotriester method (12–14) under the following conditions: (*i*) synthesis was on 16 μmol of polyacrylmorpholide T resin; (*ii*) dimers were added at 3-fold molar excess (48 μmol) over the starting resin concentration; (*iii*) the coupling reagent, mesitylenesulfonyl tetrazolide (15), was added at 3-fold molar excess over dimer; (*iv*) at degenerate positions, a mixture of appropriate monomers was added with each at 5-fold molar excess (80 μmol) and coupling reagent was added at 3-fold molar excess over total monomer concentration; and (*v*) coupling reaction time was 2 hr.

Oligonucleotide Purification. The protecting groups on the oligonucleotide mixture (20 mg of resin) were removed and the DNA was cleaved from the resin by treatment with tetramethylguanidinium 2-pyridine aldoximate overnight at room temperature, tetramethylguanidine for 4 to 5 hr at room temperature, and ammonium hydroxide at 55°C overnight. The solvent was evaporated, and the residue was dissolved in 1 ml of 10 mM triethylammonium bicarbonate ($Et_3N{\cdot}H_2CO_3$) (pH 8.0) and purified by chromatography on a 2.5 × 75 cm Sephadex G-50 column equilibrated with 10 mM $Et_3N{\cdot}H_2CO_3$. Five-milliliter fractions were collected and those containing the leading edge of the first A_{260} peak were collected, pooled, and evaporated. Residual $Et_3N{\cdot}H_2CO_3$ was removed by three successive lyophilizations from 5 ml of H_2O, and the final residue was suspended in 1 ml of 1 mM Tris·HCl, pH 7.6/0.1 mM EDTA (TE buffer) and stored at −20°C. One hundred microliters of this solution was mixed with 400 μl of glacial acetic acid, and the mixture was incubated at room temperature for 45 min to remove the 5′-dimethoxytrityl group, evaporated, and twice dissolved in 2 ml of H_2O and evaporated to dryness. The final residue was suspended in 100 μl of TE buffer and the solution was extracted with ether twice to remove dimethoxytritanol and stored at −20°C. The 17-mers in this mixture were then either purified unlabeled or labeled with ^{32}P.

Three microliters of the above 17-mer preparation was labeled with ^{32}P by using T4 polynucleotide kinase (New England BioLabs) and [^{32}P]ATP (ICN) (16). At the end of the reaction, an equal volume (10 μl) of 20% sucrose/7 M urea/0.2% $NaDodSO_4$/0.1% bromphenol blue/0.1% xylene cyanol was added, and the mixture was heated for 5 min at 37°C and separated by electrophoresis through a 20% acrylamide gel for 3.5

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Abbreviation: kb, kilobase(s).

hr at 25 mA. The gel buffer was 90 mM Tris·HCl, pH 7.9/90 mM borate/25 mM EDTA (1X TBE)/7 M urea, and the running buffer was 1X TBE. After autoradiography, the slowest migrating band, corresponding to the mixed 17-mers, was excised and the DNA was eluted in 1 ml of 0.2 M NaCl/10 mM Tris·HCl, pH 7.6/1 mM EDTA (gel elution buffer)/1% phenol overnight at 37°C. The eluate was then directly used in hybridizations.

Alternatively, unlabeled oligonucleotides in the mixture (50 μl) were separated by electrophoresis as described above and the band corresponding to the 17-mers was visualized by UV shadowing. The band was excised and eluted as above and the oligonucleotides were further purified by chromatography on a 1.0-ml column of DEAE-cellulose (Whatman DE52) equilibrated with gel elution buffer. The DNA was eluted from the column with 0.5 M NaCl/10 mM Tris·HCl, pH 7.6/1 mM EDTA and precipitated with ethanol in the presence of glycogen at 1 μg/ml. The purified 17-mers were suspended in TE buffer and used as primers in DNA sequence analyses.

Oligonucleotide Hybridizations. Restriction fragments from agarose gels (17) or phage DNA from plaques (18) were transferred to nitrocellulose and hybridized at 37°C with the labeled oligonucleotide probe (5–10 × 10^5 cpm/ml) for 20 hr according to Wallace *et al.* (14). After hybridization, the filters were washed at room temperature for 20 min with four changes of 250 ml each of 0.9 M NaCl/0.09 M sodium citrate, pH 7.2, or until unhybridized oligonucleotide was removed. Filters were exposed to Kodak XAR-5 x-ray film with a Lightning Plus intensifying screen at −70°C for 24 hr.

Clone Bank Construction and Plaque Transfer. DNA was isolated from *V. harveyi* strain B392 by sequential extraction with phenol and chloroform/isoamyl alcohol (24:1). This preparation was used for restriction enzyme digestion. A bank of hybrid Charon 13 was constructed by published methods (10). The resulting phage were pooled in groups of 500 plaques and stored at 4°C. Hybrid phage were plated with ED 8654 cells on NZ plates to give 500–1,000 plaques per plate. The plates were chilled to 4°C and phage DNA was transferred to nitrocellulose discs in duplicate (18). Phage from isolated plaques were propagated with liquid lysates according to Blattner *et al.* (10).

Subcloning and Sequence Analysis. DNA fragments were subcloned into the M13 cloning vehicles mp7 and mp8, and sequences were determined by the dideoxy method (9, 19). Gels were fixed for 10 min in 10% acetic acid and exposed to Kodak XAR-5 x-ray film at room temperature.

RESULTS AND DISCUSSION

Design and Synthesis of the Mixed Probe. The available protein sequence for the α subunit of *V. harveyi* luciferase was scanned for the sequence giving the longest possible least degenerate oligonucleotide mixture. The sequence shown (Fig. 1) is internal to the heavy delta (δ_H) chymotryptic fragment of

PEPTIDE		met	asp	cys	trp	tyr	asp	
mRNA	5′	AUG	GA$^{C}_{T}$	UG$^{C}_{T}$	UGG	UA$^{C}_{T}$	GA	3′
PROBE	3′	Ⓡ-T·AC	· CT·$^{G}_{A}$	· AC·$^{G}_{A}$	· ACC	· AT·$^{G}_{A}$	· CT	5′

FIG. 1. Design of the oligonucleotide probe for the luciferase α-subunit gene. The peptide used is internal to the δ_H chymotryptic fragment of the α subunit. Ⓡ, solid support on which the oligonucleotide was synthesized. Synthesis was in the 3′ to 5′ direction; the steps in the synthesis are separated by dots. At degenerate positions, both monomers were added. The final mixture contains all eight possible sequences and is complementary to the mRNA.

the α subunit (1, 20). This peptide sequence allows synthesis of a family of eight molecules, each 17 nucleotides long.

As shown in Fig. 1, a mixture of DNA sequences complementary to the mRNA was synthesized. During the synthesis, a mixture of the two monomers was added at each of the three degenerate positions. The rates and extents of the reactions with the monomers are known to be the same (K. Itakura, personal communication) and hence an unbiased mixture of the eight chains was expected.

Specificity of Probe Hybridization and Isolation of the Clone. Initially, the probe mixture was used to identify restriction fragments in *V. harveyi* chromosomal DNA. Hybridization was specific; the probe hybridized to a predominant band in each chromosomal digest with the restriction enzymes tested (Fig. 2*A*). No difference was observed with hybridization temperatures of 30–37°C, but wash temperatures below 20°C resulted in higher backgrounds. The probe identified a 1.85-kilobase (kb) *Eco*RI fragment, a 4.0-kb *Hin*dIII fragment, and a 5.0-kb *Bam*HI fragment. Identification of single restriction fragments of discrete size was useful in predicting the expected size of the cloned fragment.

The conditions determined by hybridization of the oligonucleotide probe to restriction fragments were used in probing the *V. harveyi* clone bank. Of the 14 pooled lysates screened, representing approximately 7,000 clones, only one pool showed positive hybridization to some of the plaques (Fig. 2*B*). DNA from the positively hybridizing phage was isolated and analyzed on agarose gels. All such phage contained two *Eco*RI fragments, one of 5.6 kb and another 1.85 kb, suggesting that they were derived from the same clone. The DNA from the gel was then transferred to nitrocellulose and hybridized with the mixed probe; only the cloned 1.85-kb *Eco*RI fragment and a fragment of the same size in a *V. harveyi Eco*RI chromosomal digest showed positive hybridization (Fig. 2*C*). Subsequent restriction analysis has shown that the 5.6-kb and 1.85-kb *Eco*RI fragments are not contiguous in the *V. harveyi* genome (data not shown).

The 1.85-kb *Eco*RI fragment was subcloned into pACYC184 and analyzed by restriction endonuclease digestion (Fig. 3). The mixed probe hybridizes to the 0.33-kb internal *Pst* I fragment (data not shown). The restriction sites shown were used to obtain fragments for further subcloning into the M13 derivatives mp7 and mp8.

DNA Sequences in the Cloned Fragment Correspond to the α-Subunit Protein Sequence. The 1.85-kb *Eco*RI fragment was subcloned into M13 mp7 in both orientations. The oligonucleotide probe was then used as a primer for dideoxy sequence analysis. The DNA sequence generated and the implied protein sequence are shown in Fig. 4*A*. The nucleotide sequence corresponds to a portion of the δ_H chymotryptic fragment, showing that at least part of *lux*A, the gene encoding the α subunit, is present. The 0.95-kb *Pst* I/*Eco*RI partial fragment (Fig. 3) was subcloned in M13 mp8. DNA sequence analysis of this fragment established the additional sequence in this region, including the site to which the mixed probe hybridized. The polarity of these sequences establishes the orientation of *lux*A.

Nicoli and Hastings (21) have shown that modification of a reactive cysteine residue on the α subunit leads to luciferase inactivation. The reactive cysteine is contained in the active center tryptic peptide Phe-Gly-Ile-Cys-Arg (22). The nucleotide sequence shown in Fig. 4*A* identifies the position of this peptide. Several trypsin cleavage sites (lysine and arginine residues) surround the peptide, which is in agreement with the observation that there are several protease-labile sites on the α subunit near the active center peptide (20, 23).

The 1.3-kb *Eco*RI/*Sal* I fragment was subcloned into M13 mp8 and the sequence was determined from the *Sal* I site (Fig.

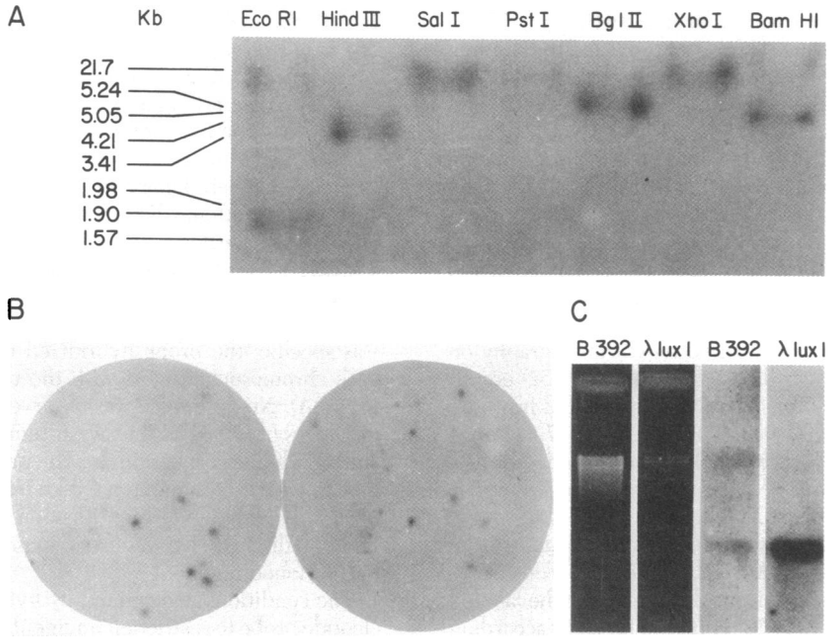

FIG. 2. Hybridization with the oligonucleotide probe. (*A*) Hybridization to restriction endonuclease-digested chromosomal DNA. Markers are *Eco*RI/*Hin*dIII-digested λ cI857; sizes are shown in kb. The high molecular weight band in the *Eco*RI lane is assumed to be due to partial digestion while the 0.33-kb *Pst* I fragment to which the mixed probe hybridizes does not appear. (*B*) Hybridization to duplicate plaque transfers from the secondary screening of positive clones. The plate contained approximately 200 plaques. (*C*) Hybridization to DNA from positively hybridizing phage. Gel photographs show *Eco*RI-digested B392 chromosomal DNA and DNA from the clone λlux1. Transfer of the DNA from the gel to nitrocellulose and hybridization with the probe shows that the 1.85-kb fragment hybridizes in both lanes.

4*B*). The protein sequence implied from these data is identical to the published protein sequence of the α-subunit amino terminus (5) with the exception of a glutamate → glutamine change at amino acid 17 and an alanine → arginine change at residue 23.

Location of the Luciferase β-Subunit Gene. Fig. 4*B* also shows a partial sequence from the *Eco*RI site downstream from *lux*A. This sequence agrees perfectly with the amino terminus of the luciferase β subunit (5). The sequence encoding the last three amino acids forms the *Eco*RI site used in the cloning. Thus, the gene for the β subunit, *lux*B, is closely linked to, downstream from, and transcribed in the same direction as *lux*A on the *V. harveyi* chromosome (Fig. 3). There is an open reading frame upstream from *lux*B. Based on the size of the α subunit and the location of the amino-terminal methionine codon, this open reading frame should correspond to the carboxyl-terminal coding region of *lux*A.

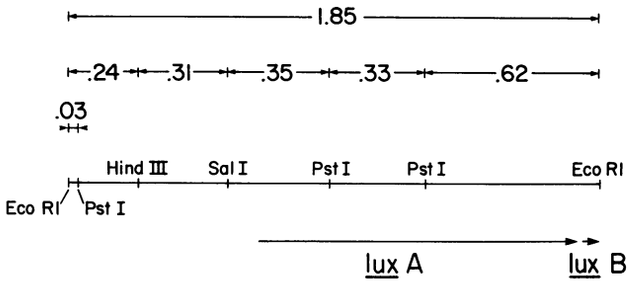

FIG. 3. Restriction map of the 1.85-kb *Eco*RI fragment and organization of the luciferase genes on the fragment. Distances between restriction sites are in kb. *lux*A and *lux*B refer to the genes encoding the luciferase α and β subunits, respectively. Arrows indicate the orientations of the genes.

The mixed probe hybridizes to a 4.0-kb *Hin*dIII fragment (Fig. 2*A*) that must contain the intact genes for both subunits. We have now cloned this fragment.

Possible Regulatory Signals Preceding *lux*A and *lux*B. Comparison of the DNA sequences upstream from the *lux*A and *lux*B structural genes reveals several homologies (Fig. 4*B*). The sequence 5′-A-A-G-G-A-A-A-3′ is centered at eight and nine nucleotides upstream from the start codons of the α- and β-subunit genes, respectively. This sequence corresponds to the consensus ribosome binding site in *E. coli* (24–26). We note two further regions of homology between the two genes located upstream from the consensus ribosome binding site. We do not yet know whether these sequences play a role in gene expression.

The Gene-Duplication Hypothesis. Based on homologies between the amino-terminal protein sequences of the two subunits, Baldwin *et al.* (5) proposed that the luciferase genes have arisen by duplication of an ancestral gene. These findings support this hypothesis and suggest that the duplication was tandem. Comparison of the nucleotide sequence in the amino-terminal coding region of *lux*A and *lux*B reveals considerable homology between the two genes; 66% of the nucleotides are identical over the first 13 codons. The homologous sequences upstream from the amino termini of the structural genes may also reflect the duplicative event.

Studies of these clones and the sequences of the genes encoding bacterial luciferase will enable us to understand the organization and expression of bacterial bioluminescence at the molecular level. Furthermore, the use of mixed-sequence oligonucleotide probes provides a generally useful way to isolate genes from organisms in which standard genetic techniques are poorly developed. This approach should be especially powerful in bacterial systems that have small numbers of genes and a relatively simple genome.

A

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 Pst I
CT GCA GCT ATC GTA TTG CCG ACT GCC CAT CCT GTT CGA CAA GCA GAA GAC GTA AAC CTA CTG GAT CAA ATG TCA AAA GGA CGA TTC CGT TTT
GA CGT CGA TAG CAT AAC GGC TGA CGG GTA GGA CAA GCT GTT CGT CTT CTG CAT TTG GAT GAC CTA GTT TAC AGT TTT CCT GCT AAG GCA AAA

   ala ala ile val leu pro thr ala his pro val arg gln ala glu asp val asn leu leu asp gln met ser lys gly arg phe arg phe

GGT ATT TGT CGC GGT TTG TAC GAT AAA GAT TTT CGT GTC TTT GGT ACA GAC ATG GAT AAC AGC CGA GCC TTA ATG GAC TGT TGG TAT GAC
CCA TAA ACA GCG CCA AAC ATG CTA TTT CTA AAA GCA CAG AAA CCA TGT CTG TAC CTA TTG TCG GCT CGG

gly ile cys arg gly leu tyr asp lys asp phe arg val phe gly thr asp met asp asn ser arg ala leu met asp cys trp tyr asp

                    δH                  gly thr asp met asp asn  X  arg lys leu met asp cys trp tyr asp
                                                                                 mixed probe
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B

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lux A   CGCTTGAAAGCGGAAATTGAAAGCCGTACGCCAGAAATGGCTTAGGTCTTATCGTAATACCAACAAATAAGGAAATGTT ATG AAA TTT GGA AAC TTC CTT CTC
        GCGAACTTTCGCCTTTAACTTTCGGCATGCGGTCTTTACCGAATCCAGAATAGCATTATGGTTGTTTATTCCTTTACAA TAC TTT AAA CCT TTG AAG GAA GAG

                                                                                        met lys phe gly asn phe leu leu

lux B   CTATGAAGCTATTCCAGTCTGATGTGATGCCATATCTCAAAGAAAAACAGTAATTAATATTTTCTAAAAGGAAAGAGAC ATG AAA TTT GGA TTA TTC TTC CTC
        GATACTTCGATAAGGTCAGACTACACTACGGTATAGAGTTTCTTTTTGTCATTAATTATAAAAGATTTTCCTTTCTCTG TAC TTT AAA CCT AAT AAG AAG GAG

                                                                                        met lys phe gly leu phe phe leu

lux A   ACT TAT CAG CCA CCT GAG CTA TCT CAG ACC GAA GTG ATG AAG CGA TTG GTT AAT CTG GGC
        TGA ATA GTC GGT GGA CTC GAT AGA GTC TGG CTT CAC TAC TTC GCT AAC CAA TTA GAC CCG

        thr tyr gln pro pro glu leu ser gln thr glu val met lys arg leu val asn leu gly

lux B   AAT TTT ATG AAT TC
        TTA AAA TAC TTA AG

        asn phe met asn ser
```

FIG. 4. DNA sequences within the cloned fragment. (*A*) Sequence generated by using the oligonucleotide probe as the primer on the M13 mp7 clone containing the entire 1.85-kb *Eco*RI fragment. The sequence of the opposite strand is from the central *Pst* I site to the right in the 0.95-kb *Pst* I/*Eco*RI partial fragment subcloned in M13 mp8 and identifies the sequence through the probe site. Also shown is a part of the δ_H fragment protein sequence aligned with the corresponding region on the clone. (*B*) Comparison of the 5'-noncoding regions and the amino-terminal coding sequences of *lux*A and *lux*B. Centered at eight and nine nucleotides upstream from the amino-terminal methionine codons of the α- and β-subunit genes are candidate ribosome binding sites. At parallel positions further upstream, additional regions of homology are identified.

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